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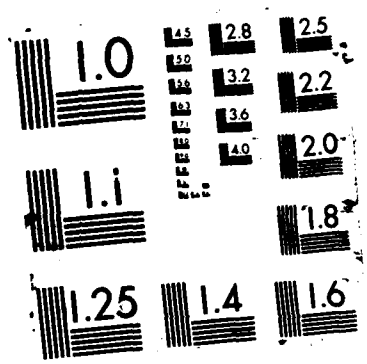
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RED CELL STORAGE STUDIES

FINAL REPORT

APRIL 15, 1987

STEPHEN B. SHOHET, M.D.

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The efforts undertaken during the performance of this contract were focused in 5 major areas: First, we established a strong correlation between in vitro red cell deformability measurements and in vivo red cell survival measurements. Second, we found that a red cell deformability defect could be induced by an increase in membrane instability secondary to a calcium:calmodulin interaction which may occur in stored cells. Third, in collaborative studies, we found that appreciable membrane vesiculation occurs during in vitro storage under blood bank conditions. Fourth, we studied the antibody-mediated accumulation of gamma-globulin on red cells and some of the mechanism of this process both with regards to the so-called "senescent" antigen, and the anti-Gal antigen. Fifth, we re-examined the assumption that cell age is directly related to cell density, and concluded that a simple correlation of cell density and cell age is not tenable.

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RED CELL STORAGE STUDIES

The efforts undertaken during the performance of this contract were focused in five major areas: First, we established a strong correlation between *in vitro* red cell deformability measurements and *in vivo* red cell survival measurements. Second, we found that a red cell deformability defect could be induced by an increase in membrane instability secondary to a calcium : calmodulin interaction which may occur in stored cells. Third, in collaborative studies, we found that appreciable membrane vesiculation occurs during *in vitro* storage under blood bank conditions. Fourth, we studied the antibody-mediated accumulation of gamma globulin on red cells and some of the mechanism of this process, both with regard to the so-called "senescent" antigen and the anti-Gal antigen. Fifth, we re-examined the assumption that cell age is directly related to cell density, and concluded that a simple correlation of cell density and cell age is not tenable.

The first area of our activity was the performance of red cell survival and ektacytometry correlation studies which we initiated at the beginning of this Proposal. At the end of the first year, we had completed 12 survivals. We eventually completed somewhat over 60 of these survivals with correlative measurements of osmotic gradient ektacytometry. With this larger number, we were able to able to demonstrate an increasingly close correlation of cell survival with cell deformability as measured in the ektacytometer. This correlation corresponded, in particular, to the region of the ektacytometric tracing which reflects surface area. From this, we concluded that the major ektacytometric correlate of *in vivo* survival of *in vitro* storage cells is the amount of reserve surface area which is preserved during cell storage. These data are seen in Figure 1, and show a correlation coefficient of approximately 0.8%. (The raw data are corrected for the slight shift in survival for stored verus fresh samples.)

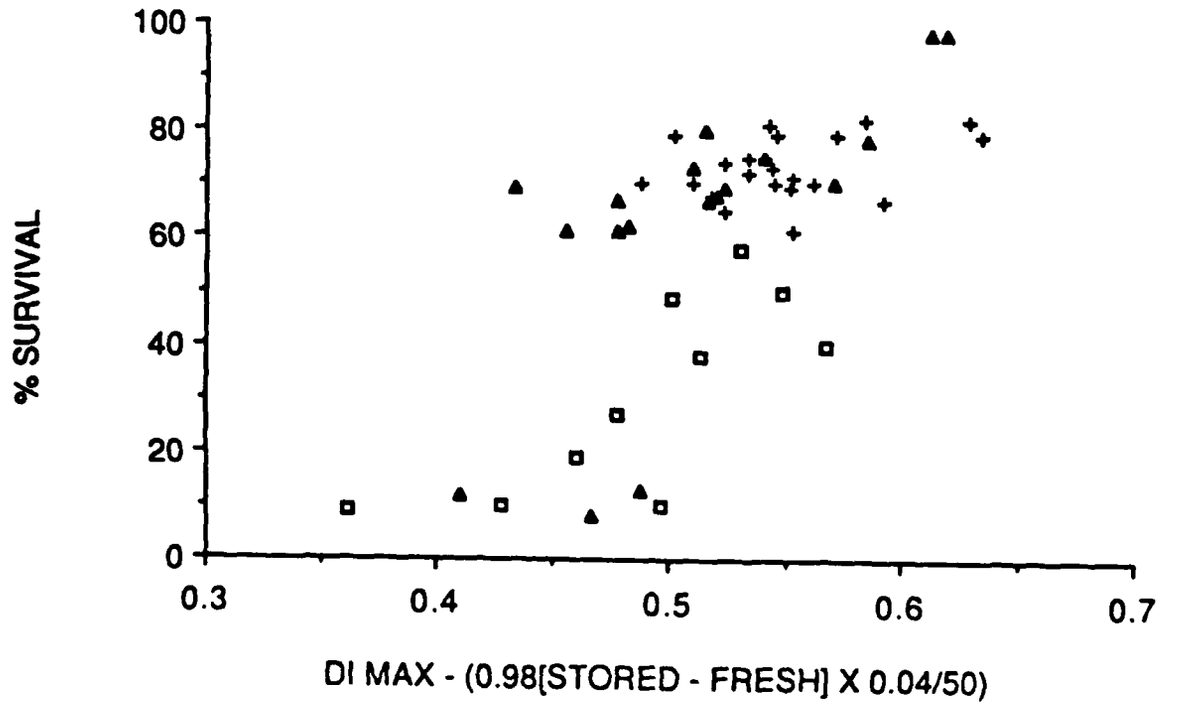


FIGURE 1. CORRELATION OF PERCENTAGE 24-HOUR *IN VIVO* SURVIVAL WITH EKTACYTOMETRIC DEFORMABILITY MEASUREMENTS. The cumulative data are presented from three sources with varying survival success. Samples provided by Letterman Army Institute of Research with survival performed in San Francisco are indicated by □ ; samples provided by Dr. Ernest Beutler with survivals performed in La Jolla are indicated by ▲ ; and samples provided by Dr. Valleri with survivals performed in Boston are indicated by + . Percentage survival is plotted against the maximum deformability index (DI_{max}) which has been shown previously shown by Clark to closely represent the total surface area of the cells. The raw data have been corrected slightly to account for the difference in survival between fresh and stored samples by subtracting Δ .98 (stored - fresh) x .04/50 from each sample. The correlation coefficient for these percent survivals versus corrected DI_{max} is 0.8 for all of these determinations taken together.

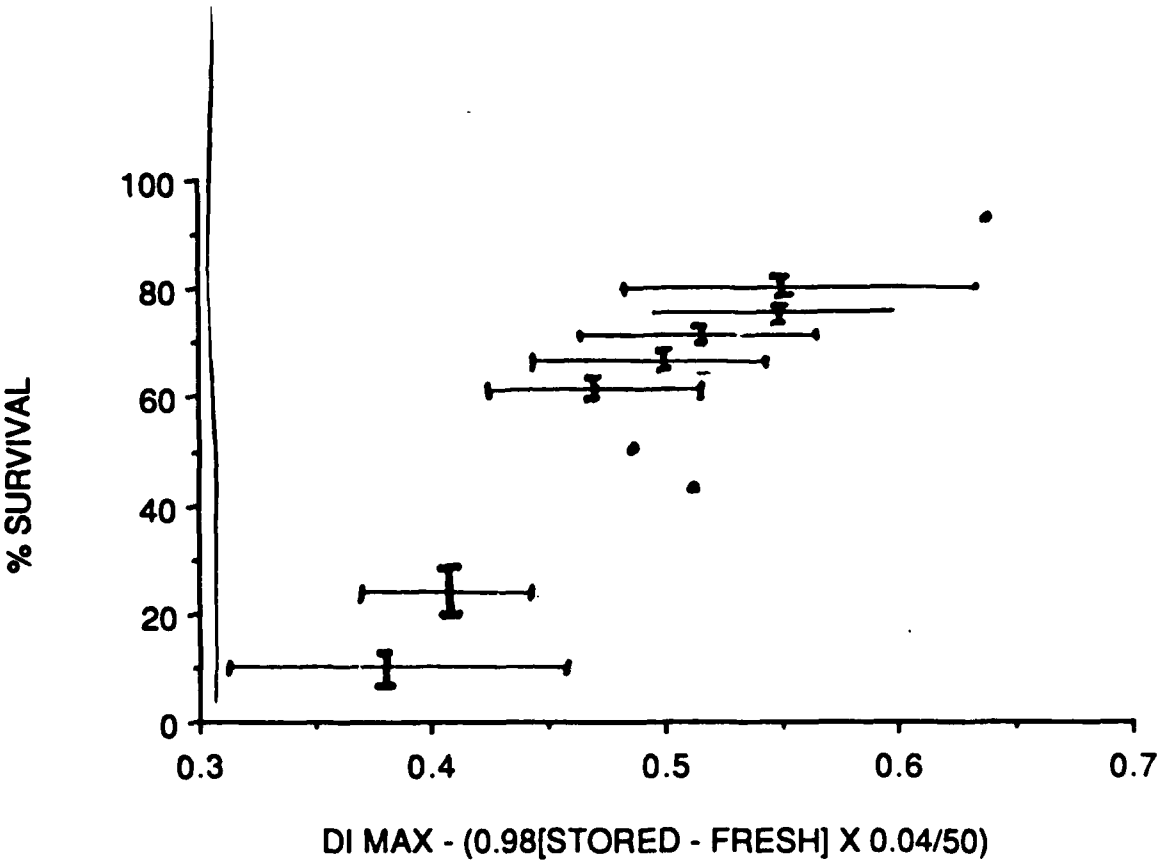


FIGURE 2. PERCENTAGE SURVIVAL VERSUS DI_{max} IN ALL SAMPLES BY 5% RANKS*. The horizontal range equals the standard deviation of the samples in each pentile; the vertical range equals the standard deviation of the percentage survivals.

(*For the second rank from the bottom, a 10% range of survivals was used in order to obtain an adequate number of samples; also, only single samples were available from some other pentiles.)

The range of variation of these data is further demonstrated in Figure 2, which provides divides the survival data into five percent increments and shows the range of the deformability measurements by the horizontal brackets and the survival data by the vertical bars.

It should be noted that these general correlations were obtained from all the pooled samples, including samples provided by Dr. Ernest Beutler in La Jolla, and Dr. Robert Valleri in Boston, as well as samples from San Francisco. It was of some interest that in terms of deformabiliy the variability of these results differed when the groups of samples provided by the three different sources were compared. However, this apparent difference may be due to the fact that the Boston and San Diego samples predominantly consisted of storage specimens with comparatively high survival percentages, whereas the San Francisco samples covered a broad range of both low and medium survivals. Perhaps because of this difference in sample source, the Boston and San Diego samples containing larger numbers for a given survival pentile show larger ranges than the San Francisco samples. Nevertheless, the data presented here were taken from all of the pooled samples in order to avoid any possible bias due to sample sources which might have been obtained from analysis of the San Francisco samples alone.

In general, from these observations, we concluded that there was a moderately strong correlation between the ektacytometric determinations of DI_{max}, which reflects cell surface area, and cell survival, after storage. Further, since we assumed that these normal donors had normal surface area prior to storage, we concluded that shortened survival, when present, was correlated with cell surface loss during storage.

The second major area of activity was prompted by the first. This consisted of our efforts to understand the mechanism of the surface area loss with an emphasis on the possible role of calcium-induced changes and calmodulin effects which may be occurring in stored cells. To this end, Dr. Takakuwa demonstrated that an interaction of calmodulin and calcium produced an increase in membrane instability with an apparent loss of membrane surface area. Again, the ektacytometer was used to define the fact that the cellular deformability defect induced by this interaction is secondary to cell surface area loss. For these studies, Dr. Takakuwa measured membrane stability and deformability of resealed ghosts prepared in the presence of calcium and physiologic concentrations of calmodulin (2-8 μmolar). For such membranes, membrane stability as measured in the ektacytometer decreased substantially with increasing concentrations of calcium (Figure 3).

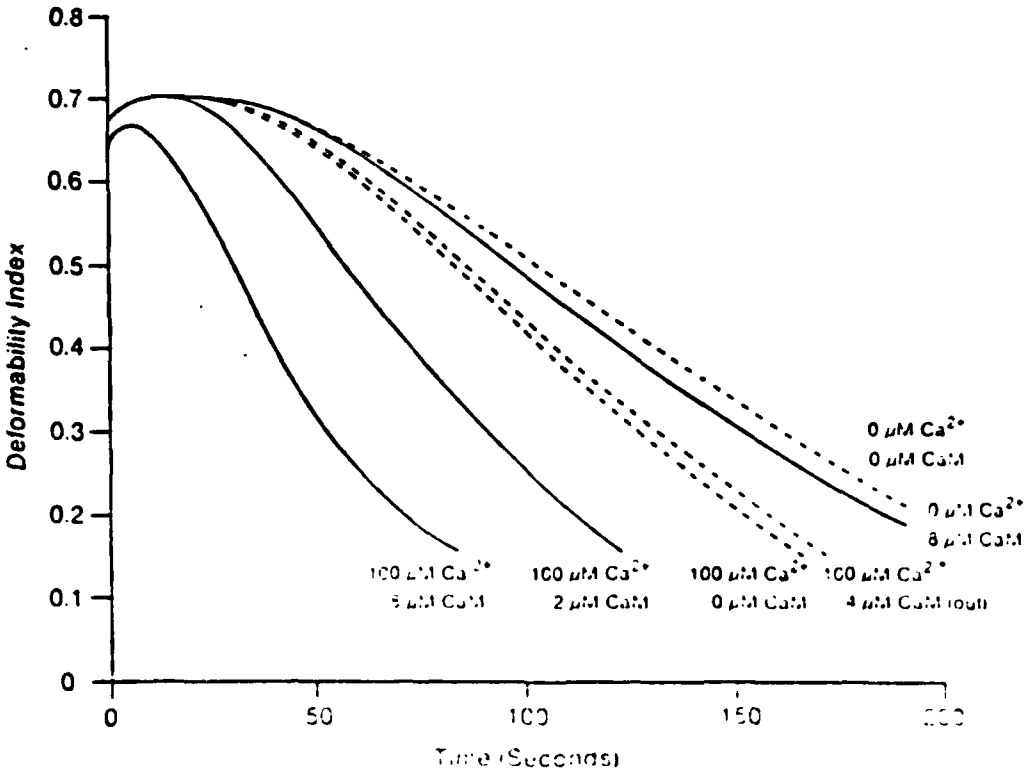


FIGURE 3. Ca^{2+}-CaM-INDUCED CHANGE IN ERYTHROCYTE MEMBRANE STABILITY. Resealed ghots were prepared in the presence or absence of Ca^{2+} (0 of 100 μM) and various concentratins of CaM (0, 2, 4 and 8 μM) and exposed to 750 dynes/cm^2 in the ektacytometer. In one experiment, 4 μM CaM was added to membrane first resealed in the presence of 100 μM Ca^{2+} (designated by 4 μM CaM [out]). This maneuver allowed the CaM to have acecss to the outer surface of the membrane, and not to the cytoplasmic side, as in all other experiments. The decline of the DI was measured as a function of time to quantitate membrane mechanical stability.

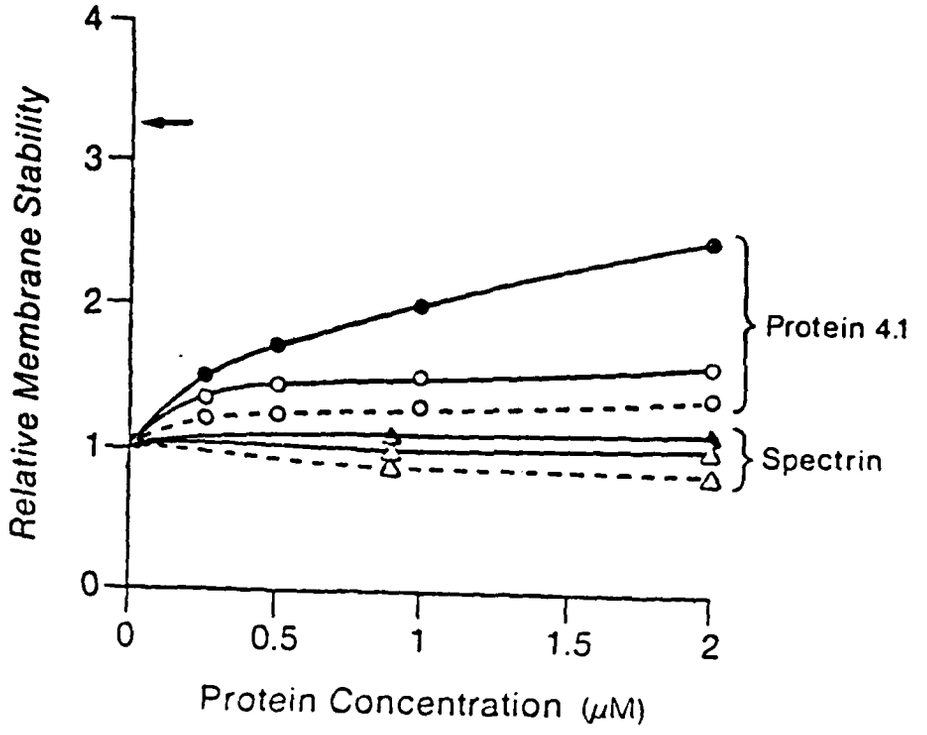


FIGURE 4. EFFECT OF PROTEIN 4.1 AND SPECTRIN DIMER ON Ca^{2+}-CaM-INDUCED CHANGE IN MEMBRANE STABILITY. Resealed ghosts were prepared in the presence of either protein 4.1 or spectrin dimer together with 2 μM alone (o–o and Δ–Δ); 500 μM Ca^{2+} alone (o–o and Δ–Δ) and 2 μM CaM and 500 μM CaM Ca^{2+} (●–● and ▲–▲). Membrane stability of the ghosts was measured and $T_{1/2}$ was obtained. The $T_{1/2}$ value obatined for ghosts for each of these Ca^{2+} and CaM concentrations in the absence of added protein was used to normalize the data. No change in relative membrane stability implies that the $T_{1/2}$ value in the presence or absence of added protein was the same; while an increase in relative membrane stability implies that the $T_{1/2}$ value was higher in the presence of added protein compared to the $T_{1/2}$ value in the absence of added protein. If the added protein completely inhibited the Ca^{2+}-CaM-induced decrease in membrane stability, we would expect the relative stability to reach a value, as indicated by the arrow, which represents the relative stability of membranes resealed in the absence of both Ca^{2+} and CaM.

Further, in the absence of calmodulin, an equivalent decrease in membrane stability was seen only when calcium concentration was increased two orders of magnitude. Also, calmodulin did not alter membrane stability in the absence of calcium. In contrast to these changes which were observed in membrane stability, membrane deformability decreased only if calcium concentrations were > 10 μmolar; and calmodulin had no effect on this calcium-induced decrease in membrane deformability. Finally, in an effort to identify the skeletal elements involved in this calcium-calmodulin-induced alteration in membrane stability, membranes were resealed in the presence of purified protein 4.1 and spectrin dimer, as indicated in Figure 4.

Only protein 4.1 and not spectrin dimer antagonized the calcium-calmodulin-induced decrease in membrane stability. These results indicated that physiologic concentrations of calcium and calmodulin could alter membrane stability, and suggested that this alteration might be due to a modulation of skeletal protein interactions involving protein 4.1. Since it is possible that small amounts of calcium interact with membrane protein constituents, even during citrate storage conditions, this series of experiments is of considerable interest. An abstract describing several of these findings was prepared and is included at the end of this report. Further, a publication more formally treating this material has now been prepared and submitted for publication and is also included with this report.

The third area of interest involved studies of membrane vesiculation which occur during storage, and which may explain the loss of surface area which, as noted above, seems to be the primary correlate of cell survival *in vitro*. In these collaborative studies, Dr. Wagner showed that appreciable membrane vesiculation occurs during *in vitro* storage under blood bank conditions and, moreover, that the addition of oxidizing agents to the storage media accelerates this membrane loss. Further, membrane vesiculation appeared to be associated with the finding of comparatively large amounts of high molecular weight aggregates of membrane proteins and reduced amounts of Band 3 proteins in the affected cells. Finally, these changes were reversible with reducing agents. Based upon these findings, it was hypothesized that oxidative damage to the red cell membrane during storage may induce the formation of intermolecular disulfide bonds within membrane

skeletal proteins. Such a reaction might be expected to disrupt protein : lipid interactions in the membrane, causing a contraction of the inner membrane leaflet and leading to echinocytic transformation and the eventual shedding of membrane vesicles. Indeed, we have observed that whenever membrane surface loss does occur, echinocytes are found to be present before that loss occurs. These studies, though not directly supported by this contract, suggested an important mechanistic hypothesis for cell surface loss damage which occurs during blood bank storage, and hence provided us with a rationale for the ektacytometric correlations which we had obtained as noted in the first section of this report.

The fourth area of interest which we completed involved studies on the possible role of an immunologic mechanism in the induction of the storage lesion in blood bank cells. The first area to report involves an experiment initiated by Dr. Galili, in which we were looking for the possibility of the accumulation of gamma globulin on stored red cells under blood bank conditions. Dr. Galili had previously demonstrated that the accumulation of gamma globulin was specific for an α-galactosyl linkage on the surface of the red cell in certain hemolytic conditions and also in senescent red cells. Accordingly, with considerable expectations, we anticipated finding similar antibodies in stored red cells. However, despite the use of a very sensitive rosetting technique to detect small amounts of gamma globulin bound to red cells, we were unable to demonstrate this phenomenon, even after eight weeks of *in vitro* storage. Accordingly, it is very likely that this immunologic mechanism is not involved in the blood bank storage lesion, and we are no longer pursuing these studies. In parallel immunologic studies conducted by Dr. Kay, we continued to try to characterize the so-called senescent cell antigen which apparently accumulates in some red cells as they mature *in vivo*. Significant progress in characterizing this antigen, localizing it to a segment of Band 3, and also showing that some proteolytic cleavage with Band 3 is necessary for its recognition, was made in this regard. In brief, a "senescent" cell antigen was found to react with red cell protein Band 3 using IgG extracted from senescent red cells (Figure 5).

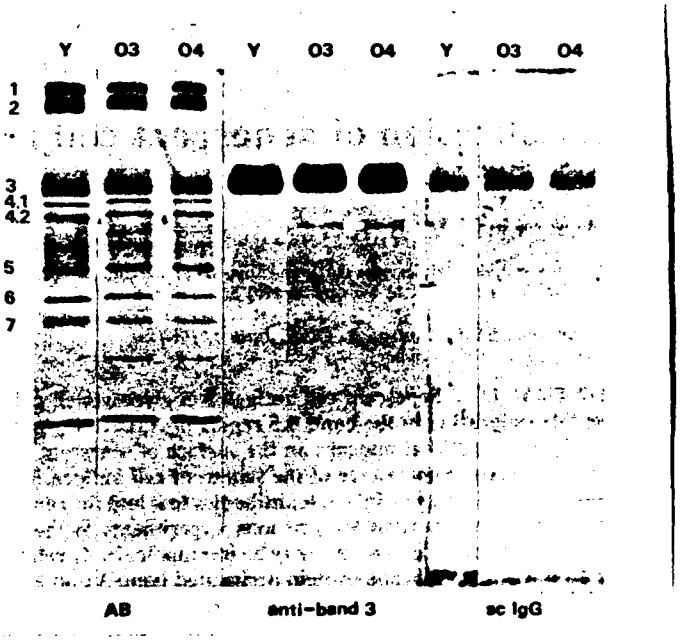


FIGURE 5. BINDING OF ANTISERA TO BAND 3 AND IgG ELUTED FROM SENESCENT CELLS TO BAND 3 AND ITS BREAKDOWN PRODUCTS. "Old" cells were separated into four bands, old fraction 4 being the densest and old fraction 3 being the second densest. The cells used for these studies represented <0.6% of the total cells. Cells were washed, and membranes were prepaed. Polypeptides were transferred from 6-25% polyacrylamide gradient gels to nitrocellulose paper and were incubated with antibodies to band 3 or IgG eluted from senescent red cells followed by ^{125}I-labeled protein A. Polypeptides were stained with amido black. both autoradiographs were exposed for the same length of time. AB, amido black; anti-band 3, antibodies to band 3; sc IgG, "senesecent cell IgG" eluted from senescent RBCs; Y, young cells; O3, old cells, fraction 3; O4, old cells, fraction 4.

However, when immunomicroscopic techniques were used to study this phenomenon in intact cells, it was found that the antibody could not bind unless the cell was pretreated with proteolytic enzymes. So the physiologic relevance of this antibody remains unknown. As noted previously, although these observations are of considerable interest in their own right, since the *in vitro* storage studies do not suggest that a primary immunologic mechanism is operating under blood bank storage conditions, we decided not to pursue these immunologic studies any further.

The fifth major area of interest involved an important experiment conducted by Dr. Margaret Clark, in collaboration with Dr. Sushana Vora, examining the basic assumption that cell age is related to cell density. This assumption, which influences the design of many experiments to study cell changes during both *in vitro* storage and *in vivo* maturation, was based upon early labeling studies, mostly in animals, which are not necessarily applicable to human red cells. Because of the importance of this assumption (and large numbers of experiments in the literature, using cell separations based upon it), we felt it incumbent upon us to re-examine it before starting any biochemical studies on gradient density separations of cells stored in blood bank conditions. Accordingly, after obtaining Experimental Committee permission, Dr. Vora conducted a ^{59}Fe labeling study with cells labeled *in vitro* and reinfused into a recipient, at the Scripps Clinic and Research Foundation in La Jolla. Blood samples were then sent to Dr. Clark here and separated by density on Stractan gradients and analyzed for radioactivity. As can be seen from the data presented in Figures 6 and 7, two surprising observations of considerable importance were made. First (Figure 6), whole blood radioactivity did not decay dramatically at 120 days, as predicted from previous models of red cell senescent destruction.

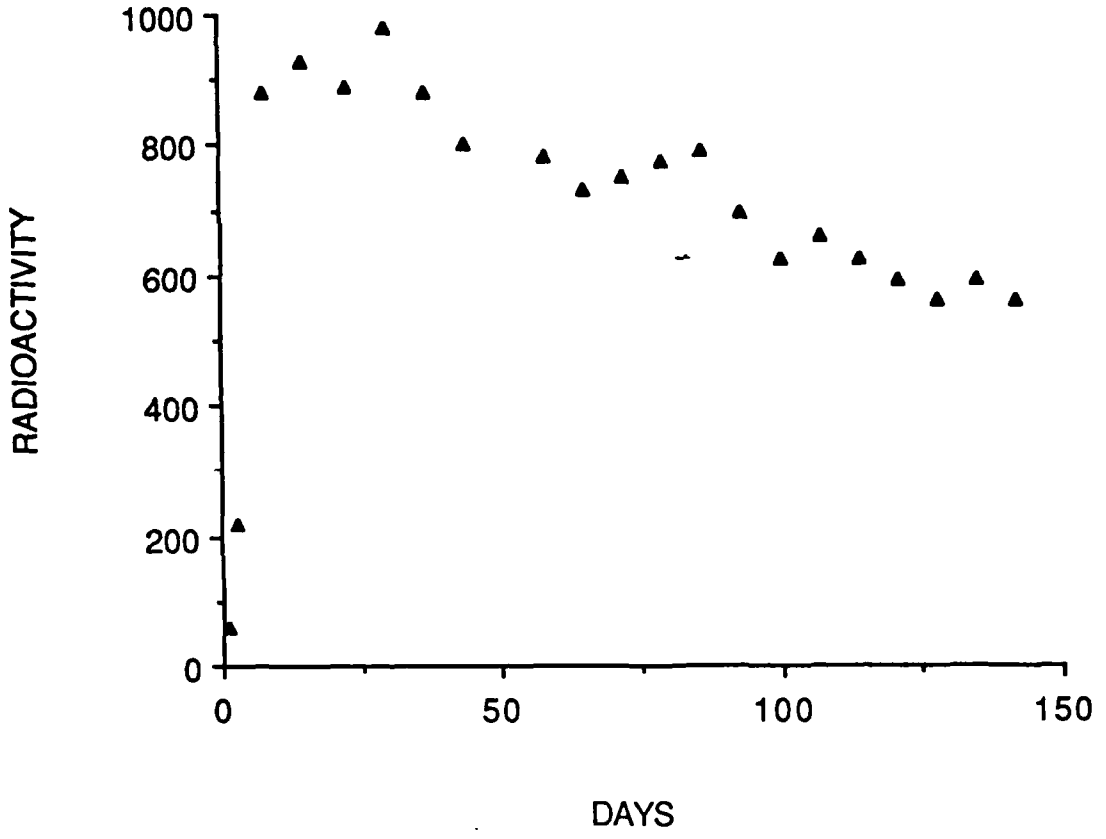


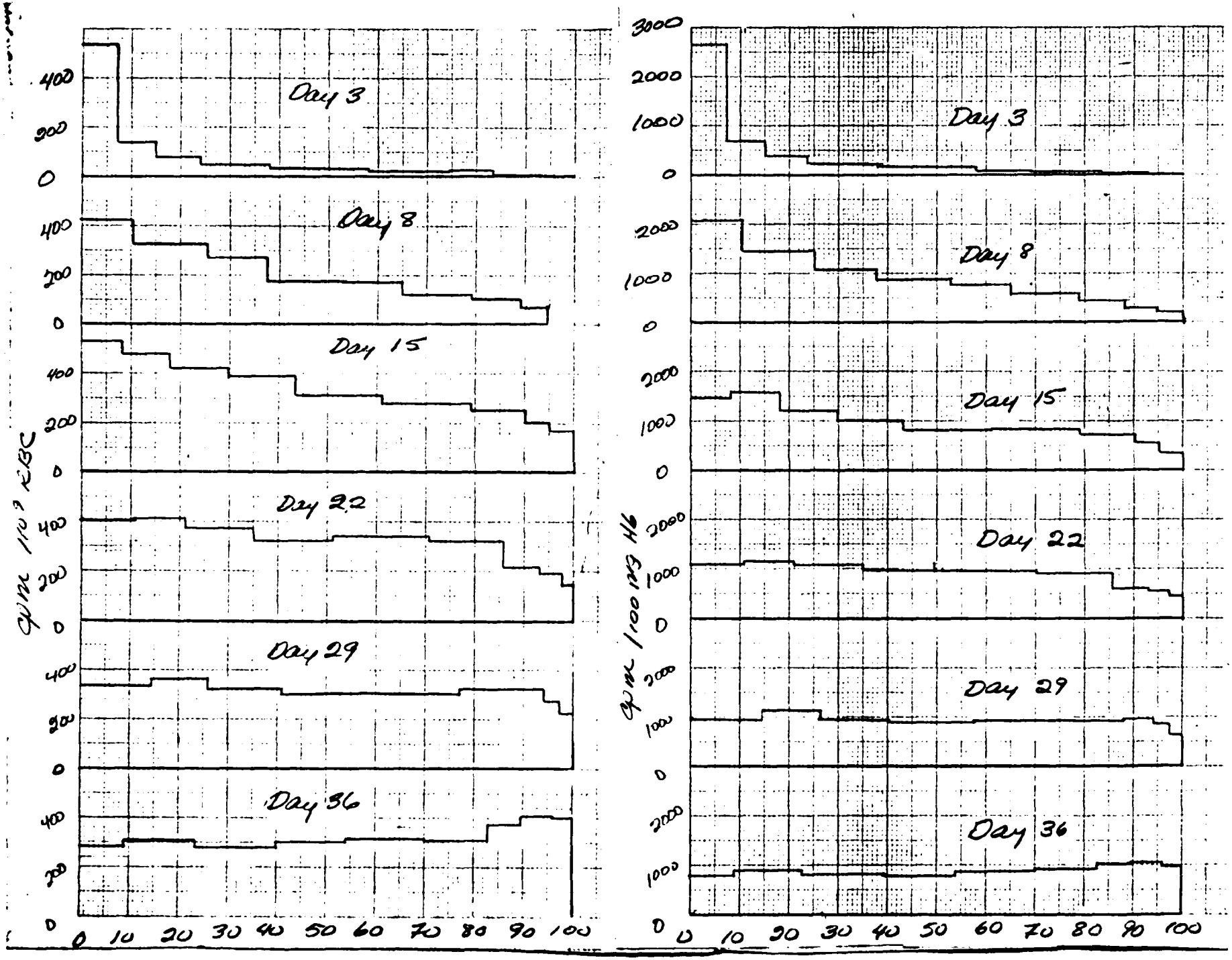
FIGURE 6. STUDIES ON CORRELATION OF CELL DENSITY AND CELL AGE; Decay of whole blood ^{59}Fe radioactivity after autologous transfusion.

Note incomplete loss of radioactivity at 120-140 days indicating label reutilization.

This observation suggested that appreciable label reutilization occurred, *in vivo*, in this experiment. Second, and much more importantly, we found that ^{59}Fe radioactivity very promptly appeared in the highest density cuts of our density gradients. As can be noted from Figure 7, even after only 8 days, significanct amounts of radioactivity were appearing in the lowest density fraction and, by day 15, these were substantial.

FIGURES 7a, b, c (see following pages). STUDIES ON CORRELATION OF CELL DENSITY AND CELL AGE; Distribution of radioactivity in density-separated cells at various times after autologous transfusion of ^{59}Fe-labled cells. Ordinates = counts; abscissae = cumulative density-fractions – in increments of approximately 10% each.

Note presence of label in heaviest fractions as early as day 8-15. This suggest that early reticulocyte remodeling is responsible for an appreciable amount of the density distribution of whole blood.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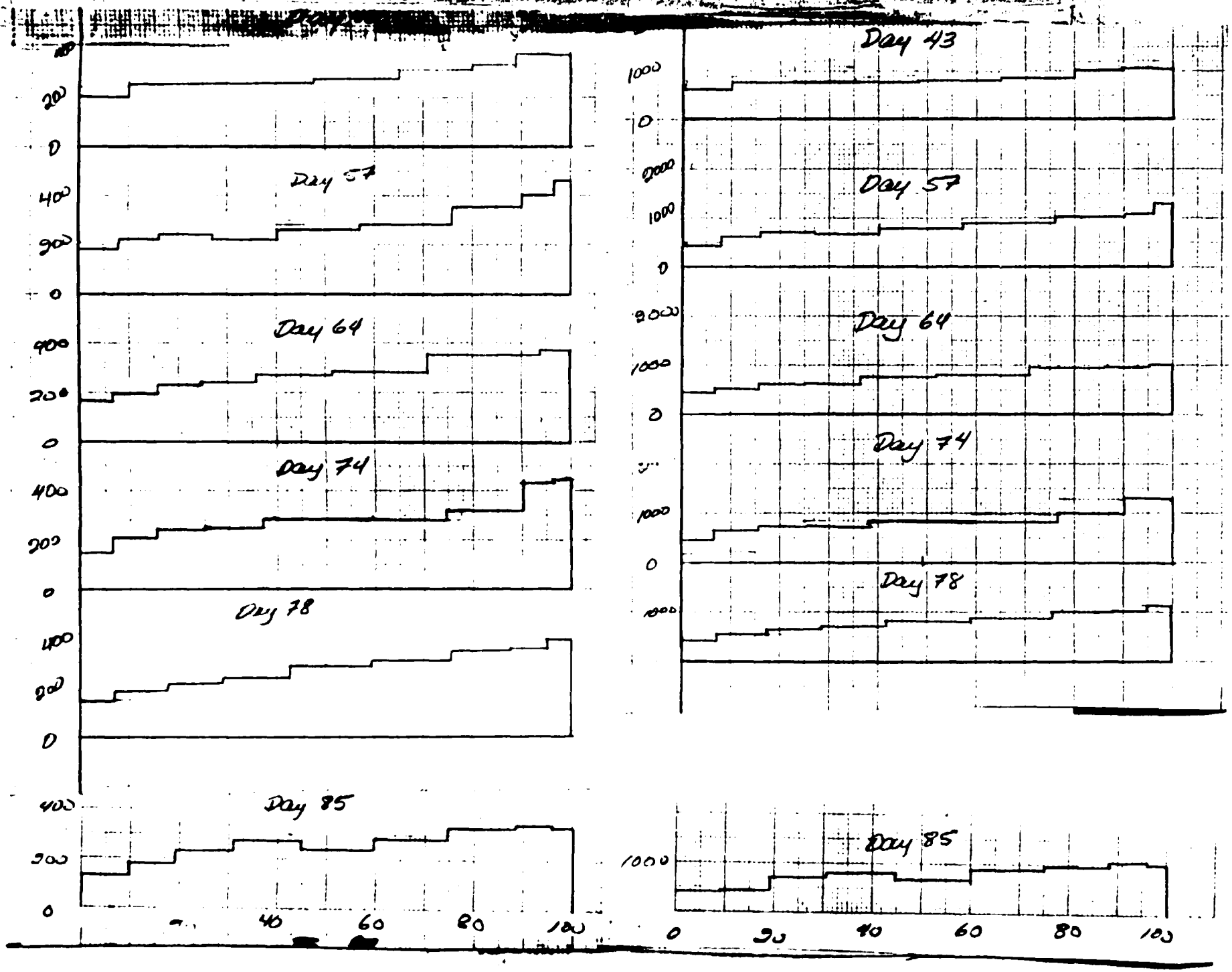
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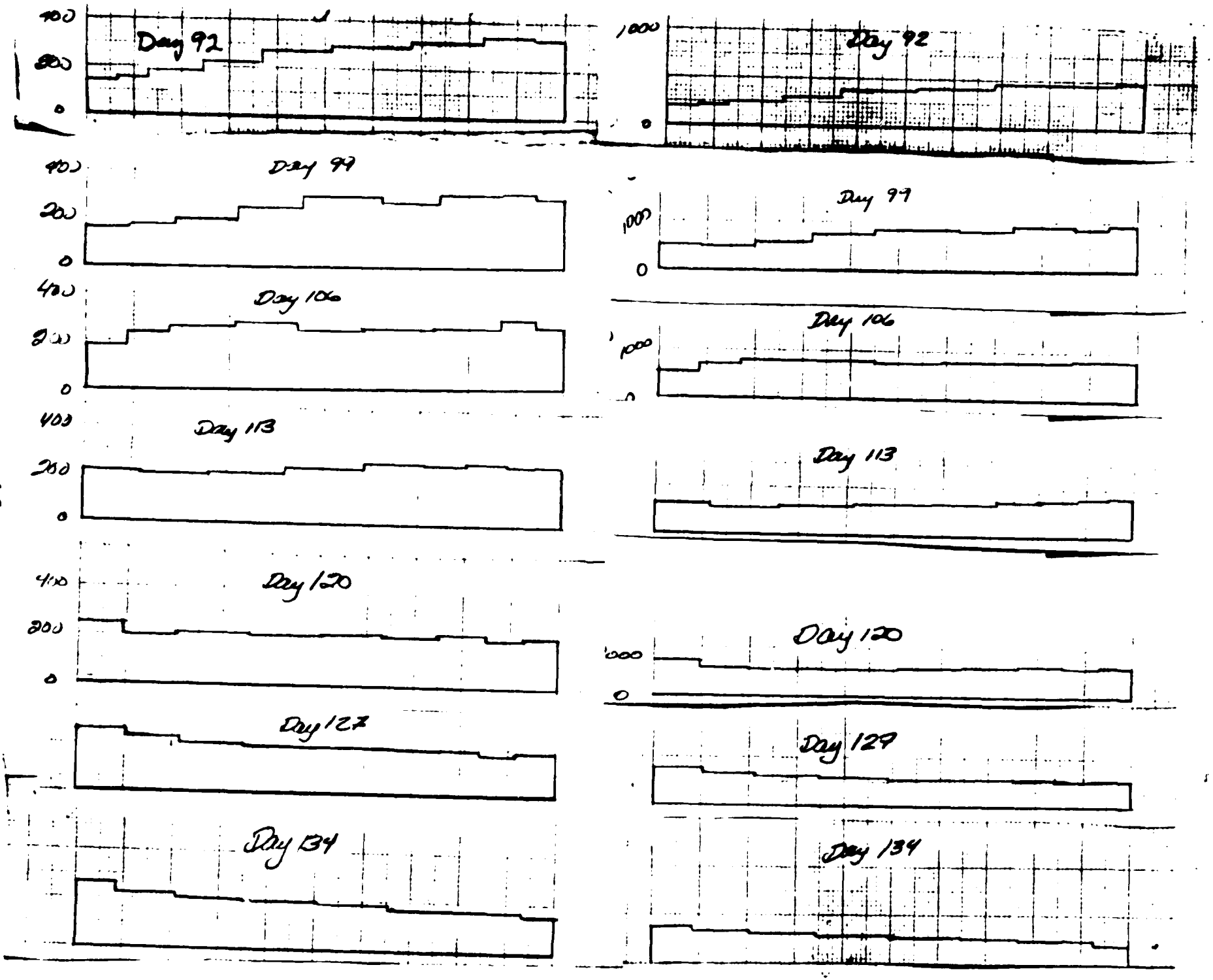
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Day 120
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Day 129
Day 134
Day 134

The finding of any significant amounts of radioactivity in the lowest fractions early in the experiment indicated that a simple correlation of cell density with cell age is no longer tenable. Indeed, it may well be that the major change in cell density occurs during the reticulocyte remodeling stage which occurs in the first few days of cell circulation. Apparently, a substantial distribution of cell densities is generated at that stage, and it is not unlikely that distribution encompasses much greater changes in cell density than any modest changes which progressively accumulate chronlogically during the lifetime of the cell. This is an important observation in terms of both our fundamental understanding of the biology of red cell maturation and in terms of any experiments designed to study red cell density changes during blood bank storage, and we feel that it will be necessary to repeat it in additioanl recipients.

The possibility that very dense cells are still subject to shortened survival remains real, however. Indeed, since dense cells are frequently associated with a reduction in surface area, and since we have already shown that such a reduction correlates well with shortened *in vivo* survival, we now need to examine the survival characteristics of dense cells directly. To this end, Dr. Clark has designed an experiment where we will directly study the survival of transfused dense cells from normal donors. Because we do not feel that we can ethically use radioactivity for such studies, Dr. Clark has devised an ingenious technique based on the old Ashby method, utilizing the NM Blood Group System to conduct this study, and has obtained Experimental Committee permission for it. We plan to approach this by transfusing NN cells into an MN recipient. In brief, we will isolate high-density NN cells from a donor, transfuse them into an MN recipient, and then periodically remove blood from the recipient to assay for circulating transfused cells.

The experiment depends upon determining the proportion of the transfused cells remaining after various times. This will be accompanied by labeling the mixed blood sample from the recipient, *in vitro*, with a fluoresceinated monoclonal antibody against the M blood group determinant and counting both the labeled (recipient) and unlabeled (donor) cells, using a Beckman-Dickinson Fluorescence-Activated Cell Analyzer. Pilot studies to prepare mixtures of NN and MM cells at the ratios expected in the experiment (1-100, down to 1-1000) and to

determine if we can reliably measure these ratios, have been successfully undertaken. We are now refining the optimal conditions for labeling the MN cells. In our most recent experiments, the background of unstained cells from a pure population of MN blood was down to 1 in 1000, which should be acceptable. Before we conduct the *in vivo* experiments, we will try a few more variations in antibody concentration, and we will determine the precision and inter-individual variations expected in these experiments. Despite its seeming mplexity, we feel that this experiment is a very direct approach to assessing the importance of cell density in cell survival and that, especially in view of the data obtained with the ^{59}Fe labeling studies, it is an essential experiment for the interpretation of the importance of any cell density changes which occur during blood bank storage conditions.

We anticipate completing this experiment without requesting additional funds from the Department of Defense for this contract.

For the protection of human subjects in these studies, the investigators have adhered to policies of applicable Federal Law 45CFR46.

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